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Novel mannose-specific adhesins and their use

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Field of the invention

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5 The present invention relates to novel mannose-specific adhesins, and variants thereof, as well as nucleic acid sequences encoding these. The invention also relates to host cells comprising one or more mannose-specific adhesins (or variants thereof), as well as foods, food ingredients, and pharmaceutical or nutraceutical compositions comprising one or more of the mannose-specific adhesins (or variants thereof) and/or comprising host cells capable of expressing these, and their use in treating, preventing  
10 or delaying bacterial infection. Further, screening methods suitable for identifying bacteria strains with probiotic properties are provided.

Background of the invention

15 Harmless commensal or even beneficial microorganisms (referred to as probiotic microorganisms) such as lactobacilli and other lactic acid bacteria commonly populate human and animal body cavities. For example, many strains of *Lactobacillus*, and related genera of lactic acid bacteria with a longstanding tradition in food industry, are present in large numbers on gastrointestinal mucosa together with other intestinal organisms (Mitsuoka, 1992 in *The Lactic Acid Bacteria in Health and Disease*, Volume  
20 I., E.B.J.B.Wood, Elsevier Science Publishing Ltd. pp 69-114; Ahme et al. J Appl Microbiol. 1998 Jul;85(1):88-94; Vaughan EE, et al. Antonie Van Leeuwenhoek. 2002 Aug;82(1-4):341-52; Reid et al. Clin Microbiol Rev. 2003 Oct;16(4):658-72; Reid et al. J Clin Gastroenterol. 2003 Aug;37(2):105-18; Mercenier et al. Curr Pharm Des. 2003;9(2):175-91). These beneficial microorganisms are believed to play an important  
25 role in human and animal health by interfering with the invasion and colonisation of potential pathogens of the gastrointestinal tract. One of the mechanisms by which interference is believed to be achieved is competitive exclusion of pathogens by the beneficial microorganisms through occupation of common receptors on the mucosal surfaces. However, little is still known about the exact mechanisms through which  
30 beneficial bacterial species become established and persist in the indigenous microflora and how they inhibit or reduce attachment and colonisation of pathogenic bacteria.

Bacterial colonisation of mucosal tissue is generally mediated by adhesins, which are proteins present on the surface of both pathogenic and beneficial bacteria. Adhesins

are thought to be responsible for the recognition and binding to specific receptors on the host cells, thereby playing a role in bacterial attachment and colonisation. In many instances, adhesins are localised on hair-like appendages of bacteria (pili or fimbriae), although they may also be directly associated with the bacterial cell surface (non-pilus adhesins) (see for example Soto and Hultgren, J. of Bacteriology 1999, Vol. 181(4), p1059-1071).

Various pathogenic Gram-negative bacteria, including enteropathogenic *Escherichia coli* (EPEC), display mannose-specific adhesion properties that play a role during the infection process. For example, the type 1 fimbriae of uropathogenic *E. coli* strains comprise the adhesin FimH, which binds to mannose ligands present on the host tissue surfaces and appears to be involved in the establishment of kidney and bladder infections (Kr  gfelt *et al.* 1990 Infect. Immun. 58:1995-1998).

Mannose-specific adhesion is a phenotype which is also found in several lactic acid bacteria. Notably, it has been shown that certain strains of *Lactobacillus plantarum* (in particular strain 299v) are capable of adhering specifically to mannoside containing sugar moieties on the surface of human epithelial cells (Adlerberth *et al.*, 1996, Applied and Environmental Microbiology, p2244-2251 and WO96/29083). It has been speculated that a proteinaceous bacterial structure is involved in this phenotype, as treatment with proteinase K could abolish the phenotype (Adlerberth *et al.*, *supra*).

Moreover, several lines of evidence show that this phenotypic feature of *L. plantarum* could be involved in the inhibition of EPEC infection by competitive exclusion (Michail & Abernathy, Pediatr. Gastroenterol. Nutr. 2002 35:350-5 and Mangell *et al.* Dig Dis Sci. 2002, 47, p511-6). This functional, phenotypic characteristic represents one of the interactions of *L. plantarum* and the human host that is functionally meaningful and experimentally testable. Various functional tests can be used to establish whether an *L. plantarum* strain possesses mannose-specific adhesion properties. Examples of such well-known functional tests are the mannose-sensitive agglutination test of yeast or erythrocytes, the direct binding of bacteria to D-mannose immobilised on agarose beads or other surfaces, cell binding (e.g. epithelial cells or cell lines) and the like.

Despite the observed phenotypic effect (mannose-specific adhesion), no molecular data are available to explain the mannose-specific adhesion properties of *L. plantarum* and therefore, it remains unknown whether indeed one or more proteins are

involved in this interaction, and if so, which protein or proteins (and which gene or genes) of *L. plantarum* is (are) involved in this phenotypic trait. Moreover, although the complete 3.3 Mbp genome sequence of *L. plantarum* strain WCFS1 has been determined (Kleerebezem *et al.*, Proc. Natl. Acad. Sci. USA, 2003, 18:1990-5), *in silico* analysis does not allow for the identification of the gene involved in this function. The inability to correlate gene sequences data with *in vivo* function is a common problem faced nowadays, as sequence homology or similarity to sequences with known function (if these are available at all) can at best provide a hypothetical function. Moreover, it is common knowledge that a significant fraction of sequenced genomes (usually about one third) cannot even be functionally annotated since these sequences are either unique, or only have homologues of unknown function. This is clearly exemplified by the finding that for the *L. plantarum* WCFS1 genome, 588 genes (~19 %) were annotated as conserved hypothetical proteins of unknown function, while 344 genes (~11 %) were annotated that do not have a homologue in public sequence databases (Kleerebezem *et al.* 2003, *supra*).

An understanding of which protein(s) are involved in determining mannose-specific adhesion in *L. plantarum* is essential for a) transferring this beneficial property to other prokaryotic or eukaryotic cells, b) improving or modulating the adhesion efficiency and/or specificity of microorganisms, c) developing pharmaceutical or nutraceutical compositions which reduce or prevent pathogen infection (e.g. vaccines) and d) for setting up easier methods for screening microorganisms, in particular bacterial collections, for the presence/absence of mannose-specific adhesion.

Microorganisms, especially probiotic bacteria, which have an improved ability to colonise human body cavities and thereby are more efficient in excluding or reducing colonisation by pathogenic microorganism, clearly have great medical value in the treatment and prophylaxis of diseases caused by pathogen infections. Similarly, the purified protein responsible for mannose-specific adhesion may in itself be of great value for the development of novel vaccines (see Wizemann *et al.* 1999, Emerging Infectious Diseases Vol. 5(3), p395-403) and therapeutic or prophylactic pharmaceuticals / nutraceuticals.

The elucidation of the gene(s) and protein(s) responsible for mannose-specific adhesion is also useful for improving the attachment and persistence of microorganisms which are used as delivery vehicles for e.g. vaccines, such as lactic acid bacteria as

reviewed by Wells *et al.* (1996 *Antonie Van Leeuwenhoek* 70: 317-330) and Pouwels *et al.* (1998 *Int. J. Food Microbiol.* 41: 155-167).

#### Description of the invention

##### 5 Definitions

“Mannose-specific adhesion” or “mannose-sensitive adhesion” refers to the ability of a cell, in particular a microorganism, especially a bacterium, to adhere to mannose comprising ligands, such as D-mannose or methyl- $\alpha$ -D-mannoside. Mannose-specific adhesion can be determined by various tests, such as the yeast agglutination  
10 test, the hemagglutination test, the binding of cells to immobilised mannose (e.g. mannose coated beads), the mannose-sensitive binding to human cell lines, and others as available in the art and as described herein. When referring to a protein or polypeptide “mannose-specific adhesion” refers to the ability of the protein to confer, or to significantly enhance, this phenotype when the nucleic acid encoding the protein  
15 is expressed in a host cell, especially a bacterial cell.

“Lactic acid bacteria” and “lactic acid producing bacteria”, is used herein interchangeably and refers to bacteria, which produce lactic acid as an end product of fermentation, such as, but not limited to, bacteria of the genus *Lactobacillus*,  
20 *Streptococcus*, *Lactococcus*, *Oenococcus*, *Leuconostoc*, *Pediococcus*, *Carnobacterium*, *Propionibacterium*, *Enterococcus* and *Bifidobacterium*.

“Probiotics” or “probiotic strain(s)” refers to strains of live microorganisms, preferably bacteria, which have a beneficial effect on the host when ingested (e.g. enterally or by inhalation) by a subject. A “subject” refers herein to a human or non-human animal, in particular a vertebrate.

25 The term “LPXTG-like anchoring motif” refers to a cell wall anchoring region commonly found in surface-anchored proteins of Gram-positive bacteria. These motifs can be used to attach proteins or hybrid proteins (e.g. covalently) to the cell surface of Gram-positive bacteria, as described by Leenhouts *et al.* (*Antonie van Leeuwenhoek* 1999, 76(1-4):367-76). The *L. plantarum* *srtA* gene encodes a sortase, which is  
30 involved in maturation and anchoring of proteins comprising an LPXTG-like anchoring motif to the cell wall (Navarre and Schneewind, 1999, *Microbial Mol. Biol. Rev.* 63:174-229).

The term "gene" means a DNA fragment comprising a region (transcribed region), which is transcribed into an RNA molecule (e.g. an mRNA) in a cell, operably linked to suitable regulatory regions (e.g. a promoter). A gene may thus comprise several operably linked fragments, such as a promoter, a 5' leader sequence comprising  
 5 e.g. sequences involved in translation initiation, a (protein) coding region and a 3' non-translated sequence comprising e.g. transcription termination sites. "Expression of a gene" refers to the process wherein a DNA region which is operably linked to appropriate regulatory regions, particularly a promoter, is transcribed into an RNA, which is biologically active, i.e. which is capable of being translated into a biologically  
 10 active protein or peptide. In one embodiment the coding sequence is preferably preceded by a nucleic acid sequence encoding a secretion signal, so that the encoded protein or peptide is secreted out of the cell. The coding sequence is preferably in sense-orientation and encodes a desired, biologically active protein or peptide.

A "chimeric" (or recombinant) gene refers to any gene, which is not normally  
 15 found in nature in a species, in particular a gene in which one or more parts of the nucleic acid sequence are present that are not associated with each other in nature. For example the promoter is not associated in nature with part or all of the transcribed region or with another regulatory region. The term "chimeric gene" is understood to include expression constructs in which a promoter or transcription regulatory sequence  
 20 is operably linked to one or more coding sequences.

A "transcription regulatory sequence" is herein defined as a nucleic acid sequence that is capable of regulating the rate of transcription of a (coding) sequence operably linked to the transcription regulatory sequence. A transcription regulatory sequence as herein defined will thus comprise all of the sequence elements necessary for initiation  
 25 of transcription (promoter elements), for maintaining and for regulating transcription, including e.g. attenuators or enhancers. Although mostly the upstream (5') transcription regulatory sequences of a coding sequence are referred to, regulatory sequences found downstream (3') of a coding sequence are also encompassed by this definition. As used herein, the term "operably linked" refers to a linkage of polynucleotide  
 30 elements in a functional relationship. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter, or rather a transcription regulatory sequence, is operably linked to a coding sequence if it affects the transcription of the coding sequence. Operably linked means



that the DNA sequences being linked are typically contiguous and, where necessary to join two protein encoding regions, contiguous and in reading frame.

The terms "signal sequence", "signal peptide" and "secretory leader" are used interchangeably and refer to a short (usually about 15-60 amino acids), continuous  
5 stretch of amino acids at the amino-terminus of secreted and membrane-bound polypeptides, which directs their delivery to various locations outside the cytosol. Signal sequences usually contain a hydrophobic core of about 4-15 amino acids, which is often immediately preceded by a basic amino acid. At the carboxyl-terminal end of the signal peptide there are a pair of small, uncharged amino acids separated by a single  
10 intervening amino acid that defines the signal peptide cleavage site (von Heijne, 1990). Despite their overall structural and functional similarities, native signal peptides do not have a consensus sequence.

The term "body cavity of a subject" is understood to mean a cavity in the body of a subject, in particular a vertebrate, that is externally accessible and that is usually lined  
15 with a mucosal surface. Examples of such cavities include the mouth, the nasopharynx, the upper and lower gastrointestinal tract, the rectum, the vagina and the non-mammalian counterparts of these mammalian cavities.

A "nucleic acid construct" is herein understood to mean a man-made nucleic acid molecule resulting from the use of recombinant DNA technology. The term "nucleic  
20 acid construct" therefore does not include naturally occurring nucleic acid molecules although a nucleic acid construct may comprise (parts of) naturally occurring nucleic acid molecules. A chimeric gene as defined above is an example of a nucleic acid construct, in particular it is an example of an expression construct. The term "vector" as used herein refers to a nucleic acid construct used for introducing exogenous DNA into  
25 host cells, such as for example bacterial cells. A plasmid vector thus at least comprises a replicon and a DNA segment that may be used for insertion of the exogenous DNA into the vector by recombinant techniques, preferably without interfering with the plasmids capability to replicate. Vectors usually comprise further genetic elements to facilitate their use in molecular cloning, such as e.g. selectable markers, multiple  
30 cloning sites and the like (see below). An "expression vector" as used herein refers to any cloning vector, in which a promoter and other regulatory elements are present to enable transcription of inserted exogenous DNA (e.g. one or more chimeric genes) when the expression vector is present in a suitable environment. The expression vector

may be a plasmid-based vector. A "shuttle vector" refers to a plasmid vector that is capable of replication and stable maintenance in at least two different host organisms, e.g. two organisms of different species or different genera. For this capability the shuttle vector may rely on a single broad host-range replicon but usually a shuttle  
5 vector will comprise different replicons for different host-organisms or different groups of host-organisms.

A "host cell" or a "recombinant host cell" are terms referring to a new individual cell (or organism) arising as a result of at least one nucleic acid molecule, especially comprising a gene encoding a desired protein, having been introduced into said cell.  
10 The host cell differs from naturally occurring cell in that they comprise one or more nucleic acid molecules, which have been introduced by either recombinant DNA techniques or by "natural" DNA transfer techniques, such as bacterial conjugation, followed by selection of recombinants at a molecular, a phenotypic and/or a functional level. The recombinant host cell may thus comprising a (man made) nucleic acid  
15 construct within its cell(s), in particular one or more chimeric genes. The host cell preferably contains the nucleic acid construct as an extrachromosomally (episomal) replicating molecule, or alternatively and more preferably, integrated into its genome. The latter has the advantage of greater genetic stability of the introduced DNA. It is immaterial by what method the nucleic acid construct is introduced into the host cell(s).  
20 Suitable transformation methods for introducing nucleic acid constructs into cells (prokaryotic or eukaryotic cells), such as e.g. electroporation, are available to a skilled person. Alternatively, the recombinant host cell may comprise one or more additional nucleic acid molecules, which were introduced by "natural" DNA transfer methods, such as bacterial conjugation, followed by targeted selection of cells in which the  
25 nucleic acid molecule(s) are introduced (and either integrated into the genome by e.g. homologous or non-homologous recombination or present on extrachromosomally replicating molecules, such as plasmids). Such a host cell is distinguished from a natural occurring cell by either the presence of additional copies of a specific gene (e.g. a change from 1 copy to more than one copy) compared to the non-recombinant cell or  
30 by the presence of one or more genes which were not previously present in the non-recombinant cell. In both cases, the introduced copy or copies of the gene(s) leads to a significant change of the phenotype of the host cell. If no copy was present in the non-recombinant cell, the conjugative transfer leads to the desired gain-of-function

phenotype (e.g. mannose-specific adhesion) due to the expression of the transferred gene. If one or more copies were already present, the additional copy (or copies) leads to an enhancement of the desired function (e.g. enhanced mannose-specific adhesion) due to the enhanced production of the protein encoded by said gene. The recombinant host cell may be a eukaryotic or prokaryotic cell, in particular a microorganism (referred to as a "recombinant microorganism"), especially a bacterium (referred to as a "recombinant bacterium").

"Food-grade" micro-organisms are in particular organisms, which are considered as not harmful, when ingested by a human or animal subject, and which are acceptable as food ingredient.

The term "selectable marker" is a term familiar to one of ordinary skill in the art and is used herein to describe any genetic entity which, when expressed, can be used to select for a cell or cells containing the selectable marker. Selectable marker gene products confer for example antibiotic resistance. Genes conferring resistance to antibiotics such as kanamycin, rifampicin, erythromycin, actinomycin, chloramphenicol, tetracyclines, or other antimicrobial compounds like nisin and lactacin F are generally known in the art. The term "reporter" may be used interchangeably with marker, although it is mainly used to refer to visible markers, such as green fluorescent protein (GFP). Selectable markers may be dominant or recessive and they may further be bidirectional.

The term "homologous" when used to indicate the relation between a given (recombinant) nucleic acid or polypeptide molecule and a given host organism or host cell, is understood to mean that in nature the nucleic acid or polypeptide molecule is produced by a host cell or organisms of the same species, preferably of the same variety or strain. If homologous to a host cell, a nucleic acid sequence encoding a polypeptide will typically (but not necessarily) be operably linked to another (heterologous) promoter sequence or, if applicable, another (heterologous) secretory signal sequence and/or terminator sequence than in its natural environment. Such regulatory sequences may also be homologous to the host cell. In this context, the use of only "homologous" sequence elements allows the construction of "self-cloned" genetically modified organisms (GMO's) (self-cloning is defined herein as in European Directive 98/81/EC Annex II; see also below herein). When used to indicate the relatedness of two nucleic acid sequences the term "homologous" means that one

single-stranded nucleic acid sequence may hybridise to a complementary single-stranded nucleic acid sequence. The degree of hybridisation may depend on a number of factors including the amount of identity between the sequences and the hybridisation conditions such as temperature and salt concentration as discussed later.

- 5        "Stringent hybridisation conditions" can also be used to identify nucleotide sequences, which are substantially identical to a given nucleotide sequence. Stringent conditions are sequence dependent and will be different in different circumstances. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T<sub>m</sub>) for the specific sequences at a defined ionic strength and pH. The
- 10       T<sub>m</sub> is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridises to a perfectly matched probe. Typically stringent conditions will be chosen in which the salt concentration is about 0.02 molar at pH 7 and the temperature is at least 60°C. Lowering the salt concentration and/or increasing the temperature increases stringency. Stringent conditions for RNA-DNA hybridisations
- 15       (Northern blots using a probe of e.g. 100nt) are for example those which include at least one wash in 0.2X SSC at 63°C for 20min, or equivalent conditions. Stringent conditions for DNA-DNA hybridisation (Southern blots using a probe of e.g. 100nt) are for example those which include at least one wash (usually 2) in 0.2X SSC at a temperature of at least 50°C, usually about 55°C, for 20 min, or equivalent conditions.
- 20       The term "substantially identical", "substantial identity" or "essentially similar" or "essential similarity" means that two peptide or two nucleotide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default parameters, share at least a certain minimal percentage of sequence identity, as defined herein below. GAP uses the Needleman and Wunsch global alignment algorithm to align two
- 25       sequences over their entire length, maximizing the number of matches and minimises the number of gaps. Generally, the GAP default parameters are used, with a gap creation penalty = 50 (nucleotides) / 8 (proteins) and gap extension penalty = 3 (nucleotides) / 2 (proteins). For nucleotides the default scoring matrix used is nwsgapdna and for proteins the default scoring matrix is Blosom62 (Henikoff &
- 30       Henikoff, 1992).

In this document and in its claims, the verb "to comprise" and its conjugations is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded. In addition, reference to an element

by the indefinite article "a" or "an" does not exclude the possibility that more than one of the element is present, unless the context clearly requires that there be one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one".

Sequences according to the invention

5       The present inventors were able to identify and isolate the nucleic acid sequence (SEQ ID NO: 2) from *L. plantarum*, which encodes a protein (SEQ ID NO: 1) responsible for mannose-specific adhesion of *L. plantarum*. This protein is referred to as "mannose-specific adhesin". This is the first mannose-specific adhesin isolated and characterised from a gram-positive bacterium. Knock-out mutants of *L. plantarum*  
10   WCFS1 (lacking a functional copy of the mannose-specific adhesion gene) completely lost the ability to agglutinate yeast in the yeast agglutination test. In contrast, overexpression of the mannose-specific adhesion gene in the wild type WCFS1 strain resulted in a significant enhancement of the mannose-specific agglutination of the recombinant strain. The experiments prove that the mannose-specific adhesin is  
15   responsible for conferring mannose-specific agglutination and adherence to cells comprising mannose molecules on the cell surface.

      The mannose-specific adhesin is 1010 amino acids in length and comprises several repeated domains at the N- and C-terminal parts (residues 120-220, and residues 700-915, respectively). According to signalP-HMM prediction, the amino acid  
20   sequence has a predicted N-terminal signal peptide for secretion by the Sec-dependent pathway, from residue 1 to 45 (<http://www.cbs.dtu.dk/services/SignalP>). Moreover, at the C-terminal the motif LPQTN is present, which is an LPXTG-like anchoring motif typically found in gram-positive cell-wall anchored surface proteins. This motif comprises a proteolytic cleavage site recognised and cleaved by the sortase enzyme (or  
25   surface protein transpeptidase), in *L. plantarum* encoded by the *srtA* gene (SEQ ID NO: 3 and SEQ ID NO: 4). However, enzymes performing similar function are also found in other gram-positive bacteria (see Navarre and Schneewind, 1999, supra).

      Homology searches reveals domain-specific similarity of mannose-specific adhesin (lp\_1229) to several surface proteins of different lactobacilli and staphylococci,  
30   and includes a protein of *Lactobacillus reuteri* that has been shown to be involved in mucus binding properties displayed by this species (Roos and Jonsson, Microbiology. 2002, 148:433-42). The sequence similarities to *L. reuteri* are limited to the C-terminal repeat region of the lp\_1229 mannose-specific adhesin. Residues 265-515 have strong

similarity to a domain of large multi-domain surface proteins of *Staphylococcus aureus* (NCBI codes Q8NUJ3, Q8VQ99, Q99QY4) and *S. epidermidis* (NCBI code Q8CMU7; hemagglutinin protein), which also have a signal peptide, LPxTG-anchor and very large repeat regions. This domain is a member of the concanavalin A-like lectin/glucanase family (Interpro code IPR008985; structure superfamily code SSF49899), for which several 3-dimensional structures are known. Members of this family are found in a wide range of species, but exhibit the common property of reversibly binding to specific complex carbohydrates. Some bacterial and fungal beta-glucanases have this Con A-like domain, and carry out the acid hydrolysis of beta-glucans found in microorganisms and plants. There are many Con A-like domains found in proteins involved in cell recognition and adhesion. Hence, the presence of a carbohydrate-binding domain and additional similarity to a mucus-binding protein corroborate the role of this lp\_1229 mannose-specific adhesin in interactions with the host or host-derived molecules.

In one embodiment, the invention provides isolated mannose-specific adhesin proteins and nucleic acid sequences encoding these. Provided is the mannose-specific adhesin of SEQ ID NO: 1 and the nucleic acid sequence encoding it, SEQ ID NO: 2. The protein or nucleic acid (DNA or RNA) may be either isolated from natural sources (such as various *L. plantarum* strains), or may be synthetically made *de novo* using chemical amino acid or nucleic acid synthesis. The mannose-specific adhesin, or functional variants thereof, may also be obtained from recombinant host cells overexpressing the mannose-specific adhesin (or a functional variant thereof), as described below.

Also provided are active variants of the mannose-specific adhesin protein of SEQ ID NO: 1, as well as nucleic acid sequences encoding these variants. Variants can be easily generated using methods known in the art, such as but not limited to amino acid substitutions or deletions (generating for example functional fragments), *de novo* chemical synthesis of peptides or mutagenesis- or gene-shuffling techniques, and the like. Variants may be tested for retention of functionality (mannose adhesion in the various assays described herein). Variants can also be identified and isolated from natural sources, such as other bacterial strains, using for example nucleic acid hybridisation techniques (e.g. Southern blot, Northern blot), and especially encompasses DNA sequences which hybridise to SEQ ID NO: 2 under stringent

hybridisation conditions (defined above). It is clear that there are many approaches in the art to isolate variants of SEQ ID NO: 1 or SEQ ID NO: 2. An alternative to the use of nucleic acid hybridisation is for example the use of specific or degenerate PCR primers, designed to hybridise to specific regions of SEQ ID NO: 2 (or its complement strand) and to amplify variants using PCR technology. Substantially identical sequences can also be identified *in silico*, by using publicly available algorithms and databases such as BLAST, FASTA and the like, using SEQ ID No's 1 or 2 (or fragments or variants thereof) as input sequence. Sequences identified in this way can then either be cloned using standard molecular biology techniques or synthesised chemically using for example an oligonucleotide synthesiser as supplied e.g. by Applied Biosystems Inc. (Fosters, CA, USA).

Variants of the mannose-specific adhesin as defined herein include peptides with amino acid sequences with at least 45%, 50%, 60%, 75%, 80%, 90%, 95% or 99% sequence identity to SEQ ID NO: 1 (as determined using the GAP program and default parameters), which retain the ability to confer or improve mannose-specific adhesion, as well as the nucleic acid sequences encoding these.

It is clear that due to the degeneracy of the genetic code various nucleic acid sequences may encode the same amino acid sequence. Thus, codons may be replaced, without changing the amino acid sequence of the protein. Similarly, some amino acids can be substituted by other equivalent amino acids without significantly changing the activity of the protein (at least not in a negative way). For example conservative amino acid substitutions within the categories basic (e.g. Arg, His, Lys), acidic (e.g. Asp, Glu), nonpolar (e.g. Ala, Val, Trp, Leu, Ile, Pro, Met, Phe) or polar (e.g. Gly, Ser, Thr, Tyr, Cys, Asn, Gln) fall within the scope of the invention as long as the mannose-specific adhesion property of the protein is not decreased by such changes. In addition non-conservative amino acid substitutions fall within the scope of the invention as long as the mannose-specific adhesion property of the protein is not decreased by such changes.

Variants of SEQ ID NO: 2 comprise nucleic acid sequences which have at least 45%, 50%, 60%, 75%, 80%, 90%, 95%, or 99% sequence identity to SEQ ID NO: 2 (as determined using the GAP program and default parameters) and encode a protein which retains the ability to bind mannose specifically, i.e. to confer a mannose-specific adhesion phenotype to the host cell in which it is expressed or, if the host cell already

shows mannose-specific adhesion, to significantly enhance the mannose-specific adhesion phenotype of a cell.

5 Variants of SEQ ID NO: 2 include nucleic acid sequences which hybridise to SEQ ID NO: 2, or variants of SEQ ID NO: 2 as described above, under stringent hybridisation conditions and encode a protein which retains the ability to bind mannose specifically, i.e. to confer a mannose-specific adhesion phenotype to the host cell in which it is expressed or, if the host cell already shows mannose-specific adhesion, to significantly enhance the mannose-specific adhesion phenotype of a cell.

10 Specific binding to mannose refers to the ability of the protein (or variant) to confer a mannose-specific adhesion phenotype to a cell in which the protein is expressed or, if the host cell already shows mannose-specific adhesion, to significantly enhance the mannose-specific adhesion phenotype of a cell. This can be tested by various tests known in the art. One suitable test for determining whether the ability to bind mannose is retained by a variant is the overexpression of the nucleic acid sequence encoding the variant in a suitable host cell, such as a knock-out mutant of *L. plantarum*,  
15 which comprises no functional mannose-specific adhesin (SEQ ID NO: 1), followed by a test suitable for determining mannose-specific adhesion of the recombinant host cell (such as the yeast agglutination test, etc., as described elsewhere herein).

20 For expression in eukaryotic cells it may be desirable to modify one or more amino acids at the N-terminal of the protein, in order to create an optimal translation initiation context in eukaryotic cells.

#### Vectors according to the invention

25 In one embodiment, the invention provides a (expression) vector comprising at least one nucleic acid sequence encoding the mannose-specific adhesin, or a functional variant thereof as described above. Thus, the nucleic acid molecule encoding mannose-specific adhesin, or a functional variant thereof, is operatively linked to at least one regulatory DNA element allowing the expression of said nucleic acid in a prokaryotic or a eukaryotic cell. Usually the nucleic acid vectors of the invention will consist of  
30 DNA. These nucleic acid vectors may be provided using techniques known per se, for which reference is made to standard handbooks such as e.g. Sambrook *et al.* (1989) and Sambrook and Russell (2001).



The nucleic acid sequence encoding the mannose-specific adhesion, or functional variant thereof, is operably linked to the nucleic acid molecules required for transcription and translation of the mannose-specific adhesin in a suitable host cell, such as a (homologous or heterologous) transcription regulatory sequence upstream  
 5 (5') of the coding sequence and (optionally) a 3' non-translated region. Suitable 3'nontranslated region (comprising a transcription terminator) may be those naturally associated with the coding sequence or may be identified as described by Ermolaeva *et al.* (J. Mol. Biol. 2000, 301: 27-33.).

Other nucleic acid elements needed for efficient transcription or translation, or  
 10 suitable to enhance transcription and/or translation, may be operably linked.

Optionally, the putative N-terminal secretion signal sequence of amino acids 1-45 of SEQ ID NO: 1 may be replaced by a different signal sequence or may be removed completely. Alternatively, the mannose-specific adhesin may be operably linked to its own, natural transcription regulatory sequence and 3'non-translated sequence, that is to  
 15 say that the complete gene, as naturally present in *L. plantarum*, may be incorporated in the vector. As the complete genome sequence is available, the upstream and downstream genomic sequences of the mannose-specific adhesin coding sequence are also available to the skilled person and can be used *per se* or be used to isolate or identify similar sequences.

20 The choice of vector elements, such as for example the choice of the transcription regulatory sequence, vector backbone, selectable marker encoding sequences, origin of replication, enhancer elements, etc., depends on the host cell in which transcription and translation are to be achieved and is easily determined by the skilled person. In principle, any transcription regulatory element that is active in the host cell may be  
 25 used, and it may be homologous or heterologous to the host cell. For efficient transcription in prokaryotic cells, such as gram-positive bacteria, preferably prokaryotic transcription regulatory sequences should be used, while for transcription and translation in eukaryotic host cells preferably elements of eukaryotic origin are used. In one embodiment preferably a promoter which is homologous to the host cell is used.  
 30 For example, *L. plantarum* transcription regulatory sequences which are either constitutively active or which are inducible, e.g. induced in the gastrointestinal tract of a subject (or in a specific part of the gastrointestinal tract), may be used. Such regulatory sequences are available in the art (Kleerebezem *et al.*, *supra*). For expression

of the mannose-specific adhesin or any variant thereof in a bacterial host cell in general nucleic acid sequences from food-grade bacteria, such as lactic acid bacteria, are preferred. Strong constitutive promoters and promoters which are strongly induced following induction are especially preferred.

- 5 Other suitable constitutive promoters are the *usp45* promoter (van Asseldonk et al., 1990, *Gene*, 95: 155-160), the *nisR* promoter (de Ruyter et al., 1997, *J. Bacteriol.*, 178: 3434-3439), the *pepN* promoter (Tan et al., 1992, *FEBS Lett.*, 306: 9-16) and the promoters mentioned in de Vos and Simons in "Genetics and Biotechnology of Lactic Acid Bacteria", Gasson and de Vos, eds., pp. 52-106, Chapman and Hall, 1994. Examples
- 10 of suitable regulated promoters are the bacteriophage *31* middle promoter and *ori*-based expression system (O'Sullivan et al., 1996, *Biotechnology*, 14: 82-87), the *xylA* promoter (Lokman et al., 1994, *Mol. Gen. Genet.*, 245: 117-125), the repressor/operator system of bacteriophage *rlt* (Nauta et al., 1996, *Mol. Microbiol.*, 19: 1331-1341), the acid-inducible or acid-responsive  $F_1 F_0$ -ATPase promoter of *L. acidophilus* (U.S. Patent No.
- 15 6,242,194), the promoters of the lactose operon of *S. thermophilus* and *lac ABCDFEGX* operon of *L. lactis* (see, e.g., Simons et al., 1993, *J. Bact.* 175:5186-5175; Mollet et al., 1993, *J. Bact.* 175:4315-4324), and the salt-inducible promoters of *L. lactis* that are disclosed in U.S. Patent No. 6,140,078.

- Another suitable class of promoters are the auto-inducible promoters as described in
- 20 e.g. Kuipers et al. (1997, *Tibtech*, 15: 135-140). Non-limiting examples are the promoters from the bacteriocin gene cluster of *Carnobacterium piscicola*, the Sakaricin gene cluster from *L. sake*, as well as (presumably) the Subtilin gene cluster from *Bacillus subtilis*. According to the invention these also include promoters for similar, non-bactericidal signal proteins involved in the quorum sensing process in microorganisms, such as the
- 25 promoter for Plantaricin A from the bacteriocin gene cluster of *L. plantarum*. Reference is made to Kleerebezem et al. (1997, *Mol. Microbiol.* 24: 895-904) and the references mentioned therein. Suitable promoters used are the auto-inducible promoters from the nisin gene cluster of *L. lactis*, especially the *nisA* and *nisF* promoters, as described in the EP 0 712 935 (*nis A*) and also in De Ruyter et al. (1996, *Appl. Environ. Microbiol.* 62: 3662-3667; and Kuipers et al., *supra* and the references given therein). These promoters
- 30 have already been used for the controlled (over-) expression of homologous and heterologous polypeptides (such as the *gusA* reporter gene from *E. coli*, *pepN*, and the lytic genes *lytH* and *lytA*) in lactic acid bacteria and for the nisin-induced expression in

heterologous hosts such as *L. helveticus* and *Leuconostoc lactis*. Further suitable promoters for use in the nucleic acid molecules of the invention are reviewed by de Vos (1999, Int. Dairy J. 9:3-10; 1999, Curr. Opin. Microbiol. 2:289-295).

The vector may further comprise one or more genes encoding selectable markers, such as genes encoding antibiotic resistance, heavy metal resistance or the ability to utilise certain sugars. As in one embodiment host cells comprising the vectors are ingested by human subjects, all genetic elements used are preferably food grade, such as sugar utilisation genes as markers or food grade auxotrophic markers. Examples of sugar utilisation genes are *xyIRAB* from *Lb. pentosus* (Posno et al., 1991, Appl. Environ. Microbiol. 57: 2764-2766), *levA* from *Lb. plantarum* (Wanker et al., 1994, Appl. Environ. Microbiol. 60: 1401-1413) or *Lb. casei* (Wanker et al., 1995, Appl. Microbiol. Biotechnol. 43: 297-303) and *scrA/scrB* from *P. pentosaceus* (Leenhouts et al., 1998, Appl. Microbiol. Biotechnol. 49: 417-423). Examples of suitable food grade auxotrophic markers are tRNA (gln) of *L. lactis* (Dickely et al., 1995, Mol. Microbiol. 15: 839-847) and *alr* of *L. lactis* or *Lb plantarum* (Bron et al., 2002, Appl. Environ. Microbiol. 68: 5663-5670).

Likewise, the vector may comprise a nucleic acid sequence encoding a sortase enzyme, preferably *srtA* of *L. plantarum*, which is co-transferred and co-expressed in the host cell. If the host cell already produces a functional sortase enzyme, i.e. a sortase enzyme which enables correct biogenesis of the mannose-specific adhesin of SEQ ID NO: 2 or a variant thereof, it is not necessary to co-express a sortase enzyme in the host. For example for expression in other lactic acid bacteria or other gram-positive bacteria most likely the introduction of mannose-specific adhesin alone will be sufficient to modify the phenotype of the cell as desired. However, if the host cell does not comprise a functional sortase enzyme, co-expression of a functional sortase may be required, as sortase is required for cross linking of the protein to the cell-wall of the host cell. It is irrelevant whether the sortase gene is introduced together with the mannose-specific adhesion (e.g. on one vector or on separate vectors) or is introduced independently, as long as the mannose-specific adhesion can be cross-linked to the cell wall of the host cell so that it can mediate mannose-specific adhesion of the host cell. Obviously, where the protein is to be secreted, for example for manufacturing compositions comprising the (purified) protein or for *in vivo* secretion in the gastrointestinal tract, cell wall anchorage is not desired and can be prevented by

choosing a host which does not mediate cell-wall anchorage (e.g. a functional sortase deficient host cell) or by modifying or deleting the LPXTG-like motif which is part of the mannose-specific adhesin sequence. In such instances, the existing signal peptide may be replaced by a suitable secretion signal (see above).

5 For other uses the localisation of the protein may not be of importance. For example, if the protein is localised within the cell or within a specific cellular compartment, it may be isolated by cell lysis and optionally subsequent purification steps prior to making a suitable pharmaceutical or nutraceutical composition.

10 Bacteria often comprise natural plasmids, which have the ability to be transferred between bacteria by conjugal transfer. Such plasmids or derivatives or parts thereof are often utilised as cloning and expression vectors. *Lactobacillus plantarum* species often harbour several natural plasmids (Ruiz-Barba et al. 1991) of which some have been characterised and sequenced (Bates and Gilbert, 1989, Danielsen, 2002, Eguchi et al., 2000, Kanakeo et al., 2000, Leer et al., 1992, Skaugen, 1989, Vujcic and Topisirovic, 15 1993). It is understood that the use of such plasmids or vectors derived therefrom may also be used as vectors for expressing the mannose-specific adhesin (or variant thereof) in a host cell according to the invention.

Also fusion proteins comprising the mannose-specific adhesin, or a fragment or variant thereof which retains the ability to bind mannose, linked in frame to another 20 coding sequence (or part thereof) is an embodiment of the invention. Fusion proteins comprising various functional domains can be generated by in frame fusion of the nucleic acid sequences encoding the domains, so that upon transcription and translation in a host cell a fusion protein is generated. In this way, different functional domains can be combined in one protein, which may be advantageous. For example, domains of 25 biologically active proteins (or whole proteins), such as proteins with known therapeutic properties, may be added so that these are displayed on the cell surface of the host cell.

In another embodiment one or more biologically active proteins and the mannose-specific adhesin according to the invention are co-expressed in a single host 30 cell, so that the host cell may be used as a vehicle to deliver these proteins to the location where the host cell binds, for example the human intestine. Co-expression may be achieved by various means, for example co-transformation of several vectors or a single vector comprising two genes, or the vector may comprise one or more nucleotide

sequences encoding different (or identical) proteins under control of a single regulatory sequence, providing bicistronic or multicistronic transcripts. The biologically active protein(s) may in this way be secreted by the host cell, while the mannose-specific adhesin binds the host cell to receptors found on the epithelial cells of a subject.

- 5        It is understood that the nucleic acid constructs or vectors of the invention may further comprise additional sequence elements for various functions known in the art *per se*. Such sequence element include e.g.: autonomously replicating sequences or origins of replication for extrachromosomal multiplication and maintenance of the vector; sequences for promoting integration of the vector or chimeric gene into the
- 10       host's genome; sequences for homologous recombination; sequences for site-specific integration and/or recombination; selection marker genes; reporter or indicator genes; sequences for promoting conjugation or other means of transfer of genetic material etc.

#### Host cells according to the invention

- 15       In another aspect of the invention a host cell comprising a nucleic acid sequence encoding a mannose-specific adhesin or variant thereof as described above is provided, or a vector as described above. The expression of the nucleic acid sequence in the host cells confers either mannose-specific adhesion to the host cell, in cases where the host cell lacks this phenotype, or enhances mannose-specific adhesion of the host cell, in
- 20       cases where the host cell already shows the ability to adhere in a mannose-specific manner. As mentioned, the nucleic acid sequence encoding the mannose-specific adhesin need not be integrated into the genome of the host cell, as long as the phenotype conferred by the expression of the nucleic acid sequence remains stable.

- It also does not matter how the mannose-specific adhesin encoding sequence is
- 25       introduced into the host cell, as long as the phenotype of the host cell is modified in the desired way and remains stable during subsequent multiplication of the host cell. There are, therefore, various methods available in the art for introducing the nucleic acid sequences or vector(s) encoding the mannose-specific adhesin or variants thereof into a host cell, such as transformation of cells or conjugative transfer between bacteria.
- 30       Methods for transforming bacterial cells are for example described in "Genetics and Biotechnology of Lactic Acid Bacteria", Gasson and de Vos, eds., Chapman and Hall, 1994. Similarly, methods for introducing recombinant DNA into fungi, plants and animals

are well known, such as particle gun transformation, protoplast transformation, electroporation, *Agrobacterium* mediated transformation and the like.

In case a host of the invention is constructed through genetic engineering such that the resulting recombinant host comprises only sequences derived from the same species as the host is, albeit in recombined form, the host is said to be obtained through "self-cloning". Hosts obtained through self-cloning have the advantage that there application in food (or pharmaceuticals) is more readily accepted by the public and regulatory authorities as compared hosts comprising foreign (i.e. heterologous) nucleic acid sequences. The present invention thus allows the construction of self-cloned *L. plantarum* and other lactobacillus hosts for food, pharmaceutical or nutraceutical applications (see also de Vos, 1999, Int. Dairy J. 9: 3-10) and such self-cloned hosts are one further aspect of the invention.

The host cell may in principle be any prokaryotic or eukaryotic cell, such as human or animal cell lines, gram positive or gram negative bacteria, fungi, viruses, or other microorganisms. In one preferred embodiment the host cell is a bacterial cell, such as a gram positive bacterium, preferably a food-grade bacterium. A host or host cells may also be a mutant, generated by classical (X-ray or chemical) mutagenesis or the host may already have been transformed previously. Other hosts are food-grade fungi and yeasts, such as bakers or brewers yeast, such as species of the genus *Saccharomyces*, *Pichia*, or *Hansenula*. In a preferred embodiment the host cell is a probiotic bacterium, which in itself has a beneficial effect when ingested by a subject.

The host cell may be a gram-positive bacterium that belongs to a genus selected from the group consisting of *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Carnobacterium*, *Bifidobacterium*, *Bacillus*, *Streptococcus*. More preferably, the host cell is selected from a Gram-positive bacterium that belongs to a species selected from the group consisting of *L. acidophilus*, *L. amylovorus*, *L. bavaricus*, *L. brevis*, *L. casei*, *L. crispatus*, *L. curvatus*, *L. delbrueckii*, *L. delbrueckii* subsp. *bulgaricus*, *L. fermentum*, *L. gallinarum*, *L. gasseri*, *L. helveticus*, *L. jensenii*, *L. johnsonii*, *L. minutis*, *L. murinus*, *L. paracasei*, *L. plantarum*, *L. pontis*, *L. reuteri*, *L. sakei*, *L. salivarius*, *L. sanfrancisco*, *Lactobacillus* ssp., *Pediococcus* spp., *Carnobacterium* spp., *C. piscicola*, *B. subtilis*, *Leuconostoc mesenteroides*, *Leuconostoc lactis*, *Leuconostoc* ssp., *B. bifidum*, *B. longum*, *B. infantis*, *B. breve*, *B. adolescentis*, *B. animalis*, *B. gallinarum*, *B. magnum*, and *B. thermophilum*.

As variants of the nucleic acid sequence may be obtained from any other organism, the nucleotide sequence encoding the mannose-specific adhesion may be homologous or heterologous to the host cell. In one embodiment the host cell originally lacks the ability to bind mannose specifically and the transfer of one or more copies of the adhesin according to confers this novel phenotype onto the host cell. The modified host cell thereby has acquired a number of novel, testable properties, namely it can bind mannose (e.g. mannose coated agarose beads), it can bind to cells comprising mannose (e.g. oligomannose chains exposed on the cell surface), e.g. human colonic cell lines and it can agglutinate mannose comprising cells, such as yeast cells or erythrocytes in a mannose-specific manner. A positive score in these tests indicates that the adhesin protein is functionally exposed on the outside of the modified cell. This is an indication that the cell also has novel properties which are useful for a variety of pharmaceutical and/or nutraceutical uses.

In another embodiment, the host cell already possesses the ability to bind mannose. For example the wild type *L. plantarum* strain WCFS1 already has this ability, as demonstrated in the tests used. However, it was surprisingly found that the introduction of at least one further copy of the adhesin coding sequence results in a significant improvement of the strain's of mannose-specific adhesion. It is thus possible to significantly enhance the phenotype by introducing additional functional copies of the mannose-specific adhesin into host cells. Such modified host cells have thus improved properties compared to the unmodified cells, as can be determined using the same test, as further described below. A host cell comprising one or more additional copies of the gene can be easily distinguished from an unmodified host cell using common molecular methods, such as PCR, hybridisation, molecular markers, etc. Also quantitative reverse transcriptase PCR (RT-PCR) or Northern blots may indicate the mRNA abundance. It is presumed that the additional copy or copies enhance the phenotype by displaying more mannose-specific adhesin proteins on the cell surface. Hence, a similar effect may be expected when the expression of a single copy is increased. Quantitative RT-PCR may therefore be used to quantify transcript levels and to select host cells which produce more mRNA transcripts of the mannose-specific adhesin (or variant) gene. In this way existing strain collections may be screened for strains with increased transcript levels, which may as a result show an enhanced mannose-specific adhesion phenotype. This may also be done following for example

mutation of strains or following transformation as described above or conjugation between strains, whereby a copy of the mannose-specific adhesion encoding gene may be transferred from one strain to another. Especially strains which produce at least 10%, 20%, 30%, preferably at least 50, 70, 90 or 100% more mannose-specific adhesin mRNA transcript (or of a variant thereof) and which additionally show a newly acquired or significantly improved mannose-specific adhesion phenotype in at least the yeast agglutination test are an embodiment of the invention.

Following introduction of at least one sequence encoding a mannose-specific adhesin according to the invention the hosts or host cells (1) express, or are able to express (e.g. upon induction), the mannose-specific adhesion sequence according to the invention, (2) expression either confers or enhances the mannose-specific adhesion properties as testable in the test described. Preferably, the cell is capable of surviving passage through a subject's gastrointestinal tract and preferably has an improved capability of adhering to and colonizing a mucosal surface lining a subjects gastrointestinal tract compared to the non-modified cell. Further, administration of the cells or compositions comprising these preferably, reduces, prevents or delays infections caused by pathogens, especially bacterial pathogens which are members of the Enterobacteriaceae, such as e.g. *E. coli*, *Enterobacter*, *Klebsiella*, *Proteus*. In particular the cells or compositions of the invention reduce, prevent or delay infections caused by pathogenic bacteria comprising type 1 fimbriae, such as e.g. *Salmonella*, *Closteridium*, *Campylobacter* and *Streptococcus*.

It is in this respect irrelevant by which precise mechanism pathogen infection is prevented, reduced or delayed following administration of the recombinant host cells according to the invention. The administered cells of the invention may for example compete for mannose-containing binding-sites with the pathogen or they may compete for growth limiting substrates, or lead to a decrease in translocation of pathogenic or potentially pathogenic bacteria over the intact epithelial surface, it may inhibit or reduce adherence of pathogenic bacteria to the epithelial cell surface, it may influence the immune system or decrease inflammatory damage, or any combination thereof, etc.

It is understood that any derivatives of the (modified) host cells described herein, which retain the desired phenotype, such as obtained through bacterial multiplication/growth are also encompassed herein.



### Functional tests according to the invention

There are a number of known test which are suitable for determining whether a cell, especially a bacterial cell, displays mannose-specific adhesion properties. All test measure mannose binding. The use of one or more of these test will allow to determine whether a cell which has been modified by introducing at least one mannose-specific adhesin encoding nucleic acid sequence according to the invention has acquired a mannose-specific adhesion phenotype (while lacking this phenotype before) or has acquired a significantly enhanced mannose-specific adhesion phenotype compared to the phenotype displayed prior to modification.

A cell is defined to display mannose-specific adhesion properties, i.e. has the ability to bind mannose, if it agglutinates yeast cells in the yeast agglutination test (see below) at the highest bacterial cell density at which agglutination can be detected by light microscopy. Preferably agglutination of yeast cells occurs in a mannose-specific manner as may be established by inhibition of agglutination by e.g. methyl- $\alpha$ -D-mannoside (see below). Maximal cell densities at which yeast agglutination can still be assessed using light microscopy are approximately  $6 \times 10^9$  colony forming units per ml in the final agglutination assay (see below). In analogy, yeast agglutination efficiency can be quantitatively expressed as the mean agglutination titre and standard deviation, which can also be applied to other materials, like purified mannose adhesion protein or (sub)cellular fractions of mannose adhesion producing cells.

An "enhanced mannose-specific adhesion" phenotype refers to an improvement of mannose-specific adhesion of at least 20%, 30%, 40% or 50% or more of the agglutination titre in the yeast-agglutination test.

### Yeast agglutination test

The "Yeast agglutination test" is a test suitable for testing mannose-specific adhesion of cells, especially microorganisms. The cell-wall of *Saccharomyces cerevisiae* comprises mannose containing polysaccharides (mannans). In this test *S. cerevisiae* cells are contacted with cells to be tested (especially bacteria), at various concentrations, in the presence or in the absence of a selected monosaccharide, one of which is mannose. Bacterial strains are grown overnight in appropriate media, washed and suspended in 0.1 culture volume PBS (pH 7.4). The cell number was recorded by plating out tenfold dilutions of the bacterial suspensions on appropriate agar plates and determining CFU/ml after 48 hours growth at 37°C. 50  $\mu$ l of twofold dilutions of the

bacterial suspensions in PBS were prepared in microtitre plates (96-well u-shaped, Greiner bio-one, Alphen a/d Rijn, The Netherlands). 50 µl PBS (pH 7.4) or PBS with methyl- $\alpha$ -D-mannopyranoside (final concentration: 25 mM; Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) were added as well as 100 µl of 1 % (w/v)

- 5 *Saccharomyces cerevisiae* suspended in PBS after growth in malt extract medium (Oxoid, Haarlem, The Netherlands). The plate was shaken for 10 minutes at room temperature and volumes of 50 µl were taken from each well to examine agglutination by bright-light microscopy (200-fold magnification, inverted microscope Nikon Eclipse TS100). The reciprocal of the highest dilution that still resulted in visible agglutination  
10 was recorded as the agglutination titre. Mean agglutination titre and standard deviation of three independent assays were calculated. This procedure is a slight adaptation of the method previously described on pages 2244 and 2245 of Adlerberth *et al.*, *supra*).

#### Erythrocyte agglutination test (Hemagglutination test)

- The glycoproteins of erythrocytes comprise mannose and it is known that certain  
15 *E. coli* and some *L. plantarum* strains agglutinate erythrocytes in a mannose-sensitive manner. This test involves mixing various dilutions of cells to be tested with an equal volume erythrocyte suspensions, either in a normal buffer solution or in a solution supplemented with D-mannose or methyl-D-mannoside. After about 15 minutes agglutination is assessed visually, using for example a light microscope at a x250  
20 magnification. The reciprocal of the highest dilution of cell suspension which results in visible agglutination within the incubation period is recorded as the agglutination titre. If agglutination of cells and erythrocytes is significantly reduced or completely inhibited by the presence of D-mannose or methyl- $\alpha$ -D-mannoside, the cells exhibit a mannose-specific adhesion phenotype. Details of this test are found for example in  
25 Adlerberth *et al.* (*supra*).

#### Direct mannose binding test

- In this test D-mannose coated agarose beads and non-coated beads are mixed with the cells to be tested and binding is measured microscopically at about 500x magnification. If the cells adhere to the D-mannose coated beads but not to the non-  
30 coated beads the test is positive and the cells show a mannose-specific adherence phenotype. Obviously, mannose may be immobilised by other means and adherence may be assessed in other ways. Again, details can be found in Adlerberth *et al.*, *supra*.

#### Human cell binding test

A suitable cell line, which is known to comprise mannose moieties on its surface, such as human adenocarcinoma cell line HT-29, is mixed with the cells to be tested, either in the presence or absence of selected monosaccharides. The number of cells attached to at least 40 HT-29 cells is determined using interference-contrast microscopy at a magnification of about x500. The specific ability of mannose to significantly reduce adherence of the test cells to HT-29 cells shows that the test cells exhibit a mannose-specific adherence phenotype. In particular, other monosaccharides such as D-glucose, methyl- $\alpha$ -D-glucoside, L-fucose, N-acetyl-glucosamine, galactose, etc. are not able to inhibit or reduce adherence. *L. plantarum* strain 299v has been described to adhere to HT-29 cells at about 10 bacteria per cell in the absence of mannose. In the presence of methyl- $\alpha$ -D mannoside adherence was reduced by 45-73%.

These above tests are non-limiting, and alternative or modified tests can be easily devised by a skilled person.

#### Screening methods according to the invention

In a further aspect the invention relates to method, in particular screening methods, for determining whether a bacterium has probiotic properties, is capable of adhering to human cells and/or has the ability to bind mannose. The method preferably comprises determining the presence or absence of a nucleotide sequence comprised in SEQ ID NO: 2 or parts or variants. Preferably the presence of the nucleotide sequence, or more preferably its expression is indicative of probiotic properties. The bacterium preferably is a bacterial strain or (single colony) isolate from a genus selected from the group consisting of *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Carnobacterium*, *Bifidobacterium*, *Bacillus*, *Streptococcus*.

In one embodiment of the invention PCR primers and probes, as well as kits comprising these, for detecting the mannose-specific adhesin DNA and/or RNA sequences are provided. PCR primers which pair to amplify SEQ ID NO: 2 or parts or variants thereof can be synthesised based on SEQ ID NO: 2 as known in the art (see Dieffenbach and Dveksler 1995, PCR Primer: A laboratory manual, Cold Spring Harbor Lab. Press, or McPherson et al. 2000, PCR-Basics: From background to bench, first edition, Springer Verlag, Germany). PCR primers may, for example, comprise at least 18, 19, 20 or more nucleotides identical to part of SEQ ID NO: 2 (or to its

complementary strand), or they may be degenerate and not match 100% to SEQ ID NO: 2 (or its complementary strand).

Likewise, SEQ ID NO: 2, or fragments thereof, can be used as hybridisation probe. A detection kit may comprise either mannose-specific adhesin primers or probes, an associated protocol to use these and optionally other reagents required.

The primers, probes or kits may be used to a) quickly identify cells which comprise one or more nucleic acid sequence according to the invention and may as a result have a mannose-specific adhesion phenotype or b) isolate variants of the nucleic acid sequence encoding the mannose-specific adhesin according to the invention from suitable cells.

The screening method for determining whether one or more cells, especially bacteria, are capable of mannose-specific adherence involves the steps of a) determining the presence or absence, within the DNA or RNA of the cell, of at least one nucleotide sequence according to the invention, b) selecting the cell which comprises the nucleotide sequence(s) and c) optionally testing the mannose-specific phenotype of the cell using for example the yeast agglutination test described.

Step a) may for example involve PCR amplification or nucleic acid hybridisation, or fingerprinting methods, such as AFLP, RFLP, RAPD, or other methods, as known in the art. Generally, the cells are grown and harvested, DNA or RNA is isolated from them and the specific DNA or RNA is detected using direct or indirect detection methods as known. The cells screened are preferably bacteria, especially Gram-positive bacteria that belong to a genus selected from the group consisting of *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Carnobacterium*, *Bifidobacterium*, *Bacillus*, *Streptococcus*.

The screening method may also make use of protein detection methods, wherein for example bacterial collections are screened for the presence / absence of the mannose-specific adhesin protein, or variants thereof. The protein may be detected directly or indirectly, using for example immunohistochemical methods, Enzyme-linked immunosorbent assay (ELISA) or Western blotting, and other methods known in the art. The assays may use a wide variety of labels, such as radioisotopes, enzymes, fluorescers, chemilumescers, spin labels, and the like. Antibodies (e.g. monoclonal antibodies) for use in such detection assays may be raised against the mannose-specific adhesin (or a variant thereof) using known methods.

In a preferred embodiment, the expression of the nucleic acid molecule encoding the mannose-specific adhesin according to the invention confers to the host cell, or significantly improves, the ability to adhere to human cells *in vitro* (e.g. cultured cell lines) and/or *in vivo*, especially to cells found in the human gastrointestinal tract, such as epithelial cells (e.g. intestinal epithelial cells, vaginal epithelial cells, urethral epithelial cells, etc.). Especially attachment and colonisation of the cell to mucosal surface are preferably improved. Also persistence within the subject is preferably longer.

The ability to adhere can, for example, be tested using the human cell binding test above. In this test the adherence of pathogen cells (e.g. pathogenic *E. coli*, *Salmonella typhimurium*, etc.), which are known to bind to the cells in a mannose-sensitive manner, may be compared to the adherence of the recombinant host cells and to mixtures of the pathogen cells and the recombinant host cells. Preferably, addition of the recombinant host cells to the pathogen cells will significantly reduce adherence of the pathogen cells. Especially addition of the recombinant host cells prior to the addition of the pathogen may have a significant effect on reducing pathogen adherence.

*In vivo* test may be carried out in test animals, such as rats, mice, rabbits and the like. In such test compositions comprising the recombinant host cells are administered to the subject's gastrointestinal tract (e.g. orally or rectally) for several days (while not being administered to a control group), followed or concomitantly with administration of a pathogenic bacterium. Tissue samples may then be taken from the gastrointestinal tract are analysed to determine whether the prior or concomitant administration of the host cell has decreased attachment and colonisation by the pathogen.

## 25 Compositions according to the invention and their use

### a) host cell comprising compositions and methods

In a further embodiment compositions (pharmaceutical and/or nutraceutical compositions and/or foods and/or food ingredients and/or probiotics) are provided which comprise the host cells as described above and one or more pharmaceutically or physiologically acceptable carriers. How such compositions are made is generally known in the art.

Additionally, a method for producing the pharmaceutical and/or nutraceutical composition comprising a host cell, said host cell having been modified to comprising

at least one nucleic acid sequences encoding a mannose specific-adhesin of SEQ ID NO: 1, or a functional variant thereof, whereby the host cell has an acquired a mannose-specific adhesion phenotype or has an enhanced mannose-specific adhesion phenotype compared to the unmodified host cell, the method comprising the steps of

5 a) culturing the host cell in a suitable medium and b) mixing the host cell with one or more pharmaceutically (physiologically) acceptable carriers. It is clear that the host cells may be purified from the culture medium prior to mixing these with additional components.

Pharmaceutical/nutraceutical compositions will usually be used for enteral, for

10 example oral, nasal/inhalation, vaginal or rectal administration or tube feeding. Pharmaceutical compositions will usually comprise a pharmaceutical/physiologically acceptable carrier in addition to an effective amount of the host cells of the invention. An "effective amount" refers to the minimal dosage of host cells which is able to significantly reduce or prevent infection of at least one species of pathogenic

15 microorganisms, such as an enteropathogenic bacterium comprising type 1 fimbriae. The effective amount can easily be determined by a skilled person. The preferred form of the composition depends on the intended mode of administration and therapeutic, prophylactic or health-beneficial application. The carrier can be any compatible, nontoxic substance suitable to deliver the host cells to the desired body cavity, e.g. the

20 intestine of a subject. E.g. sterile water, or inert solids may be used as the carrier usually complemented with pharmaceutically acceptable adjuvants, buffering agents, dispersing agents, stabilisers and the like. The compositions may further comprise additional biologically or pharmaceutically/physiologically active ingredients, such as additional microorganisms (e.g. additional probiotic bacteria), biologically active

25 proteins, etc..

The concentration of host cells will vary, depending on the formulation and application of the composition. Preferably, the compositions will contain the host cells in amounts that allow for convenient (oral) administration of the host cells, e.g. as or in one or more doses per day or per week. In particular, the preparations may contain a

30 unit dose of the host cells. For enteral application the dose of host cells is preferably at least  $1 \times 10^6$  cfu, preferably between about  $1 \times 10^6$  -  $1 \times 10^{12}$  cfu (colony forming units) per day, more preferably between about  $1 \times 10^7$  -  $1 \times 10^{11}$  cfu/day, more preferably about  $1 \times 10^8$  -  $5 \times 10^{10}$  cfu/day, most preferably between  $1 \times 10^9$  -  $2 \times 10^{10}$  cfu/day and can be fine-

tuned empirically, without undue experimentation. The host cells are preferably live or viable cells (e.g. lyophilised cells). The effective dose may be subdivided into several smaller dosages and administered for example in two, three or more portions per day. The cells may be protected by a protective coating, in order to increase survival during passage through the stomach.

In a further embodiment dead or non-viable bacterial cells of the strain(s) are used in the above compositions, instead of or in addition to live (or viable) bacteria, as for example described in WO01/95741. The amount of dead or non-viable cells used may, for example, be equivalent to that used for live bacteria. Suitable amounts can be easily determined by a skilled person. In such compositions, the amounts of cells are counted (e.g. using a flowcytometer) or measured in a different way as known to a skilled person, as measurement as 'colony forming units' is not feasible.

The host cells may also be present in the composition in the form of viable, lyophilised (freeze dried) cells, which become active again after administration or reconstitution with liquid.

The compositions will either be in liquid, e.g. a stabilised suspension of the host cells, or in solid forms, e.g. a powder, or in semi-solid form. E.g. for oral administration, the host cells can be administered in solid dosage forms, such as capsules, tablets, and powders, or in liquid dosage forms, such as elixirs, syrups, and suspensions. The host cells can be encapsulated in gelatin capsules together with inactive ingredients and powdered carriers, such as e.g. glucose, lactose, sucrose, mannitol, starch, cellulose or cellulose derivatives, magnesium stearate, stearic acid, sodium saccharin, talcum, magnesium carbonate and the like.

In one embodiment a composition according to the invention is suitable for consumption by a subject, preferably a human or an animal. Such compositions may be in the form of a food supplement or a food or food composition, which besides the host cells of the invention also contains a suitable food base. A food or food composition is herein understood to include liquids for human or animal consumption, i.e. a drink or beverage. The food or food composition may be a solid, semi-solid and/or liquid food or food composition, and in particular may be a dairy product, such as a fermented dairy product, including but not limited to yoghurt, a yoghurt-based drink or buttermilk. Such foods or food compositions may be prepared in a manner known per se, e.g. by adding the host cells of the invention to a suitable food or food base, in a suitable

amount. In a further preferred embodiment, the host cells are micro-organisms that are used in or for the preparation of a food or food composition, e.g. by fermentation. Examples of such micro-organisms include baker's or brewer's yeast and lactic acid bacteria, such as probiotic lactic acid strains. In doing so, the host cells of the invention  
 5 may be used in a manner known per se for the preparation of such fermented foods or food compositions, e.g. in a manner known per se for the preparation of fermented dairy products using lactic acid bacteria. In such methods, the host cells of the invention may be used in addition to the micro-organism usually used, and/or may replace one or more or part of the micro-organism usually used. For example, in the  
 10 preparation of fermented dairy products such as yoghurt or yoghurt-based drinks, a food grade lactic acid bacterium of the invention may be added to or used as part of a starter culture or may be suitably added during such a fermentation.

In another aspect the invention relates to a method for (site-specific) production of a polypeptide or a biologically active substance at a mucosal surface of a subject.  
 15 The method comprises the step of administering to the subject a composition comprising the host cells as described above. As the host cells are particularly suited to attach to and colonise mucosal surfaces, they can be used effectively to deliver other biologically active substances to the mucosal surface of subject. This can be achieved by either pre-loading the cells with such substances, or by co-expressing biologically  
 20 active proteins or polypeptides in such cells, which are then produced and/or released locally at the site of host-cell colonisation. These may be membrane bound or secreted proteins or protein fragments with biological activity. In one embodiment, the polypeptide is an antigen from a human or animal pathogen. The *in situ* production of the pathogen's antigen(s) allows for the vaccination of the human or animal to which  
 25 the composition is administered. In another embodiment the polypeptide or the biologically active substance is a health-promoting factor.

#### b) protein comprising compositions and methods

In another embodiment of the invention a pharmaceutical and/or nutraceutical  
 30 composition comprising a mannose-specific adhesin, or a variant thereof, as described, and one or more pharmaceutically (physiologically) acceptable carriers is provided.



In another embodiment a method for producing a polypeptide according to the invention is provided, the method comprising the steps of:

- a) culturing a host cell which has been modified to comprise at least one (newly introduced or additional) nucleic acid sequence encoding a mannose-specific adhesin, or a variant thereof, under condition conducive to the expression of the polypeptide; and, b) recovery of the polypeptide.

Also provided is a method for producing the pharmaceutical (and/or nutraceutical) composition, the method comprising the steps a) and b) here above and further comprising the step of mixing the polypeptide with a pharmaceutically (physiologically) acceptable carrier. Prior to mixing the polypeptide with the carriers, it may optionally be purified using protein purification methods known in the art. Preferably, the polypeptide is secreted from the cultured host cell and can be easily recovered from the culture medium.

The embodiments described above for compositions comprising host cells apply analogously to compositions comprising the mannose-specific adhesin or variant(s) thereof.

#### Use of the compositions

Provided is also a method for treating or preventing a pathogen infection of the gastrointestinal tract, the urinary tract, and/or the vagina comprising administering to a patient in need of such treatment an effective amount of a composition comprising host cells as described above or comprising a suitable amount of the mannose-specific adhesin, as described. Especially, infection of a mucosal surface in a subject caused by a pathogenic bacterium which expresses type 1 fimbriae can be prevented or significantly reduced by administration of a composition according to the invention.

The host cells or mannose-specific adhesin polypeptide as described can, thus, be used for the manufacture of a medicament for the treatment or prevention (prophylactic treatment) of a pathogen infection of the gastrointestinal tract, the urinary tract, and/or the vagina, in particular for the treatment or prevention of infections of a mucosal surface caused by one or more bacterial species which express type 1 fimbriae.

In particular, the compositions may be used for the treatment, prevention or delay of infections caused by the pathogenic microorganisms as indicated above.

The following non-limiting Examples describe the identification and isolation of mannose-specific adhesin. Unless stated otherwise, the practice of the invention will employ standard conventional methods of molecular biology, virology, microbiology or biochemistry. Such techniques are described in Sambrook *et al.* (1989) *Molecular Cloning, A Laboratory Manual* (2<sup>nd</sup> edition), Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press; in Sambrook and Russell (2001) *Molecular Cloning: A Laboratory Manual*, Third Edition, Cold Spring Harbor Laboratory Press, NY; in Volumes 1 and 2 of Ausubel *et al.* (1994) *Current Protocols in Molecular Biology*, Current Protocols, USA; and in Volumes I and II of Brown (1998) *Molecular Biology LabFax*, Second Edition, Academic Press (UK); *Oligonucleotide Synthesis* (N. Gait editor); *Nucleic Acid Hybridisation* (Hames and Higgins, eds.), all incorporated herein by reference.

Description of the figures

Figure 1: Yeast mannose-specific agglutination test

Top left: only yeast; Top right: yeast cells and *L. plantarum* WCFS1; Bottom left: *L. plantarum* WCFS1 and methyl-mannoside; Bottom right: *L. plantarum* WCFS1  $\Delta$ *srtA*

Figure 2: Agglutination of *Saccharomyces cerevisiae* mediated by *L. plantarum* WCFS1 mutant strains. Mean agglutination titres (log 2) +/- SD calculated from 3 independent experiments are displayed. Wildtype (A) and *lp\_0373* mutant strains (B and C) show the same capacity to agglutinate yeast cells. In contrast, *cat*-replacement of *lp\_1229* (D) leads to a complete loss of this ability, whereas overexpression of *lp\_1229* (E) results in enhanced agglutination. A: *L. plantarum* WCFS1 wildtype; B: *L. plantarum* WCFS1 *lp\_0373::cat*; C: *L. plantarum* WCFS1 *lp\_0373* overexpression; D: *L. plantarum* WCFS1 *lp\_1229::cat*; E: *L. plantarum* WCFS1 *lp\_1229* overexpression.

ExamplesExample 1 – in silico analysis

It was found that *in silico* analysis was not suitable to identify which gene or genes of *L. plantarum* is responsible for the mannose-specific adhesion phenotype described. To illustrate this, a list of *L. plantarum* genes which display sequence homology to genes identified in other bacterial species and which in those other species potentially play a role in adherence to mucosal surfaces is listed below.

The format is given as follows: lp\_xxxx : gene name of the hit from homology search [*species of origin of the hit*]. Lp\_xxxx refers to the *L. plantarum* gene as designated by Kleerbezem et al. (2003) and EMBL database accession no. AL935263.

*L. plantarum* gene : homology hit [*species of origin of the hit*]

- lp\_0304: aggregation promoting protein [*Lactobacillus gasseri*]
- lp\_0520: autoaggregation-mediating protein [*Lactobacillus reuteri*]
- 15 lp\_1229: mucus binding protein precursor; Mub [*Lactobacillus reuteri*]
- lp\_1643: mucus binding protein precursor; Mub [*Lactobacillus reuteri*]
- lp\_1793: fibronectin-binding protein-like protein A [*Streptococcus gordonii*]
- lp\_1873: aggregation-relevant adhesin [*Streptococcus gordonii*]
- lp\_2278: autoaggregation-mediating protein [*Lactobacillus reuteri*]
- 20 lp\_2845: aggregation promoting protein [*Lactobacillus gasseri*]
- lp\_2847: aggregation promoting protein [*Lactobacillus gasseri*]
- lp\_3050: aggregation promoting protein [*Lactobacillus gasseri*]
- lp\_3059: mucus binding protein precursor; Mub [*Lactobacillus reuteri*]
- lp\_3114: mucus binding protein precursor; Mub [*Lactobacillus reuteri*]
- 25 lp\_3209: mucus adhesion promoting protein [*Lactobacillus reuteri*]
- lp\_3214: collagen binding protein [*Lactobacillus reuteri*] / mucus adhesion promoting protein [*Lactobacillus reuteri*].

This list is non-exhaustive and homology may also be due to homology in only a limited, specific domain. Such regional homology in many cases does not allow function annotation and merely indicates that certain domains are conserved.

This list illustrates the impossibility of predicting which gene or genes of *L. plantarum* are responsible for mannose-specific adhesion, despite the availability of the complete genome sequence of *L. plantarum*.

5 Example 2 – Identification of mannose specific adhesin encoding gene(s) in *L. plantarum* WCFS1

Mannose specific adhesion properties of *L. plantarum* strains were evaluated in a simple assay that is based on *L. plantarum* mediated yeast (*Sacharomyces cerevisiae*) agglutination, which can be inhibited by the addition of specific mannosides, as  
 10 described in Adlerberth et al. (1996) with slight modifications. In brief, bacterial strains were grown overnight in appropriate media, washed and suspended in 0.1 culture volume PBS (pH 7.4). The cell number was recorded by plating out tenfold dilutions of the bacterial suspensions on appropriate agar plates and determining CFU/ml after 48 hours growth at 37°C. 50 µl of twofold dilutions of the bacterial suspensions in PBS  
 15 were prepared in microtitre plates (96-well u-shaped, Greiner bio-one, Alphen a/d Rijn, The Netherlands). 50 µl PBS (pH 7.4) or PBS with methyl-α-D-mannopyranoside (final concentration: 25 mM; Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) were added as well as 100 µl of 1 % (w/v) *Saccharomyces cerevisiae* suspended in PBS after growth in malt extract medium (Oxoid, Haarlem, The Netherlands). The plate was  
 20 shaken for 10 minutes at room temperature and volumes of 50 µl were taken from each well to examine agglutination by bright-light microscopy (200fold magnification, inverted microscope Nikon Eclipse TS100). The reciprocal of the highest dilution that still resulted in visible agglutination was recorded as the agglutination titre. Mean agglutination titre and standard deviation of three independent assays were calculated.

25 Initial experiments using *L. plantarum* WCFS1 have indicated that this strain can mediate yeast-agglutination in a mannoside specific manner (Figure 1). Next to WCFS1, various *L. plantarum* strains were tested on their capacity to agglutinate *Saccharomyces cerevisiae*. Under the applied conditions reproducible agglutination titres were obtained for individual strains. In addition, yeast cells did not agglutinate  
 30 without the addition of bacteria, verifying that this process is induced by bacteria (Figure 1). Finally, it could be concluded that the yeast agglutination titres differed between the *L. plantarum* strains, showing diversity in this capacity between the strains (Table 1). Importantly, several strain lacked the capacity to agglutinate yeast cells. For

all strains that displayed yeast agglutination, this agglutination could be completely inhibited by addition of methyl- $\alpha$ -D-mannopyranoside, demonstrating a mannose-specific mechanism (data not shown).

**Table 1:** Agglutination of *Saccharomyces cerevisiae* mediated by various *L. plantarum* strains. Mean agglutination titres (log 2) +/- SD calculated from 3 independent experiments are shown. The third column provides information on the source of the *L. plantarum* strains:

<i>L. plantarum</i> strain	Mean titre (log 2) $\pm$ SD	Source; reference
ATCC 14917	6.0 $\pm$ 0.0	Pickled cabbage, ATCC
ATCC 8014	0 $\pm$ 0.0	Maize ensilage, ATCC
CIP 104440	0 $\pm$ 0.0	Human stool, ref. 1
CIP 104441	0 $\pm$ 0.0	Human stool, ref. 1
CIP 104450	0 $\pm$ 0.0	Human stool, ref. 1
CIP 104451	0 $\pm$ 0.0	Human urine, ref. 1
CIP 104452	7.7 $\pm$ 0.6	Human tooth abcess, ref. 1
299	7.3 $\pm$ 0.6	Human GI tract, ref. 2
299v	6.7 $\pm$ 0.6	Human GI tract, ref. 2
NC8	4.0 $\pm$ 0.0	Silage, ref. 3
LM3	5.3 $\pm$ 0.6	Silage, ref. 4
LP80	6.7 $\pm$ 0.6	Silage, ref. 5
LP 85-2	0 $\pm$ 0.0	Silage, NCIMB collection
WCFS1	4.8 $\pm$ 0.6	Human saliva, sequenced strain

5 References in Table 1:

- 1: Gasser and Sebald 1966, Ann Inst Pasteur (Paris) 110:261-275.
- 2: Adlerberth et al. AEM
- 3: Aukrust, T., and H. Blom. 1992. Food Res. Int. 25:253-261.
- 4: K. Thompson, K. McConville, L. McNeilly, C. Nicholson, and M. Collins, Abstr.
- 10 6th Symp. Lactic Acid Bacteria Genet. Metab. Appl., p. E5, 1999
- 5: Scheirlinck T, et al. Appl Environ Microbiol. 1989 55:2130-2137.

15 This information was matched to the *L. plantarum* genotype-diversity database that was constructed previously on basis of *L. plantarum* strain-genotyping using the *L. plantarum* WCFS1 clone-based DNA microarrays (unpublished). This matching revealed a list of 10 *L. plantarum* WCFS1 genes of which the presence / absence score in the diversity database consistently coincides with the observed phenotypic variation observed in the yeast agglutination assay. Notably, two of these genes (lp\_0373,

lp\_1229) encode LPXTG-containing surface proteins and were therefore considered highly likely candidates for the mannoside specific adhesin function.

Correct biogenesis of these LPXTG-containing surface proteins is strictly dependent on the sortase enzyme (encoded by the *srtA* gene; lp\_0514) that catalyzes the cross-linking of these proteins to the cell-wall. In contrast to several other Gram-positive species, the *L. plantarum* genome encodes only a single sortase enzyme. Previously, *srtA* mutants of *L. plantarum* have been constructed in the inventors laboratory (unpublished). Evaluation of the *srtA* mutant derivatives of *L. plantarum* strains WCFS1 and 299v in the yeast agglutination assay revealed that these mutant had completely lost the capability to mediate yeast agglutination (Figure 1). These findings strongly supported the postulated mannose-specific adhesion properties of the two (either one of the two, or both) LPXTG-containing surface proteins identified using the biodiversity database (lp\_0373, lp\_1229).

#### 15 Example 3 - Validation of lp\_1229 as the mannose-adhesin encoding gene

The experimental approach to validate the potential role of lp\_0373 and / or lp\_1229 encompassed both the construction of knock-out mutant strains and overexpression mutants in *L. plantarum* WCFS1. Mutant derivatives of *L. plantarum* WCFS1 that lack a functional copy of either lp\_0373 or lp\_1229 were constructed by double-cross over gene replacement strategy. Knock-out suicide plasmid constructions were performed with *E. coli* as cloning host. Molecular biology techniques such as DNA manipulations and transformation of *E. coli* were essentially performed according to standard procedures (Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular cloning: a laboratory manual, 2nd ed.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.). Transformation of plasmid DNA to *L. plantarum* was performed using electroporation (Ferain, T., Garmyn, D., Bernard, N., Hols, P., and Delcour, J. (1994) *Lactobacillus plantarum* IdhL gene: overexpression and deletion. *J Bacteriol* 176: 596-601.) Plasmid DNA was isolated from *E. coli* using Jetstar columns following the manufacturer's instructions (Genomed GmbH, Bad Oeynhausen, Germany).

30 Primers used in this study are listed in Table 2 and were purchased from Proligo France SAS (Paris, France). Restriction endonucleases, *Taq* and *Pfx* polymerase and T4 DNA ligase were used as recommended by the manufacturer (Gibco BRL Life Technologies, Gaithersburg, Maryland, USA; Invitrogen, Breda, The Netherlands; New

England BioLabs, Beverly, MA, USA). Plasmids used or constructed in this study are listed in Table 3.

### 3.1 Construction of lp\_0373 and lp\_1229 gene replacement mutants

*Lactobacillus plantarum* WCFS1 chromosomal DNA was used as a template to amplify the 5'- and 3'- flanking regions of the two candidate genes, using the primer combinations lp\_0372F / lp\_0372R, lp\_0374F / lp\_0374R, lp\_1227F / lp\_1227R and lp\_1230F / lp\_1230R, respectively (Table 2). If necessary, PCR-products were digested with *Bsa*HI (sites introduced in primers). These PCR-products (~0.8 kb) were ligated into the mutagenesis vector pNZ7101 after digestion with *Sma*I or *Pvu*II and *Bsa*HI, respectively. The resulting plasmids contain a chloramphenicol resistance cassette (*cat*) between the flanking regions of lp\_0373 or lp\_1229, respectively, as well as a erythromycin resistance gene. These vectors, designated pAL0373 and pAL1229, were used for stable double cross-over gene replacements in the chromosomal DNA of *L. plantarum*. Therefore, they were introduced into *L. plantarum* WCFS1 by electroporation. Integrants were selected primarily by plating on MRS agar containing 7 µg/ml chloramphenicol and double cross over candidate colonies were identified by selection of erythromycin sensitivity, as evaluated by transfer of colonies to MRS plates containing 25 µg/ml erythromycin. The anticipated double cross over gene replacement of lp\_1229 and lp\_0373 by the chloramphenicol resistance encoding *cat* gene was verified by PCR and Southern blot analysis.

### 3.2 Construction of lp\_1229 and lp\_0373 overexpression mutants

The two candidate genes lp\_0373 and lp\_1229 were amplified by PCR using *L. plantarum* WCFS1 genomic DNA and the primer combinations lp\_0373F / lp\_0373R or lp\_1229F / lp\_1229R. PCR-products of approximately 3.1 and 3.7 kb, respectively, were cloned into a pCR-Blunt vector (Invitrogen, Breda, The Netherlands) which subsequently was digested with *Xho*I and *Not*I (sites integrated in primers). Fragments consisting of lp\_0373 and lp\_1229, respectively, were subsequently ligated into expression vector pJL22. This vector contains a constitutive promoter, located within the 100bp fragment upstream of lp\_1144 of *L. plantarum* WCFS1 cloned into the pNZ273 *gusA* promoter probe vector (Platteeuw C, Simons G, de Vos WM. Appl Environ Microbiol. 1994 60:587-593). The *gusA* gene of this expression plasmid was removed by digestion with *Xho*I and *Not*I digestion and replaced by lp\_0373 or lp\_1229, which were obtained as *Not*I-*Xho*I fragments from the corresponding PCR-



BLUNT clones, yielding pAW002 and pAW005, respectively. These high-copy plasmids were transformed into *L. plantarum* WCFS1, resulting in constitutive overexpression of the genes cloned.

### 3.3 Functional analysis of mutants constructed

5       The constructed mutant derivatives of strain WCFS1 displayed no obvious changes in bacterial phenotype nor in apparent growth or viability differences as compared to the wildtype strain.

10       To investigate the importance of the two candidate genes for mannose-specific adhesion, the constructed mutant strains of *L. plantarum* WCFS1 were subsequently again tested on their agglutination capacity. Results are shown in Figure 2. Concerning lp\_0373, no involvement in mannose-specific adhesion could be concluded as both mutant strains of *L. plantarum* WCFS1 either lacking or overexpressing lp\_0373 showed the same behaviour in the agglutination assay as the wildtype strain. In conclusion, the protein encoded by this gene is not necessary for the capacity to  
15       agglutinate *S. cerevisiae*. In contrast, results obtained with the lp\_1229 knock-out and overexpression mutants in the agglutination assay establish the involvement of lp\_1229 in mannose-specific adhesion. The knock-out mutation of this gene resulted in a complete loss of the ability of *L. plantarum* WCFS1 to agglutinate *S. cerevisiae*. Moreover, overproduction of lp\_1229 resulted in a significant increase of the yeast  
20       agglutination titre. Similar to what had been observed before, this increased agglutination efficiency displayed by the lp\_1229 overproducing mutant could be completely abolished by the addition of methyl- $\alpha$ -D-mannopyranoside. These results clearly establish a direct role of the protein encoded by lp\_1229 in mannose specific agglutination of yeast cells. These results show that lp\_1229 encodes the mannoside-  
25       specific adhesin of *L. plantarum*, which is involved in the reported *L. plantarum* mediated inhibition of EPEC infection.

**Table 2:** primers used for knock-out and overexpression constructs of lp\_1229, and lp\_0373.

lp_0372F	5'-AAGGAACTTCTGATTGGGCC-3'
lp_0372R	5'-CCATTACGATGATGTTTCCC-3'
lp_0374F	5'- AATTACGGCGTCTCACTGTAGCGATTACGATG- 3'
lp_0374R	5'-CGTGTTTCTTGGAAGACTTC-3'
lp_1227F	5'-TATGCAGAACTCCATGACTG-3'
lp_1227R	5'-TAGTGTGTTTTATGCTCTCC-3'
lp_1230F	5'- ATTTATGGCGTCGTCTCCTTGGTATGGCTAAG- 3'
lp_1230R	5'-CATCACTCGACATGTCTTGC-3'
lp_0373F	5'- AAGGACAGAAGCGGCCGCGTTAGATAAAAGG GGTTAAAC-3'
lp_0373R	5'- ACGGGCCTCGAGATATTAGTCACGCCCTCGT-3'
lp_1229F	5'- AAGGACAGAAGCGGCCGCGTGCGAAAGGAT AGATT-3'
lp_1229R	5'-ACGGGCCTCGAGGCCCTACTCTTTGTGCT-3'

**Table 3:** plasmids used in this study

pNZ7101	Cm <sup>R</sup> Ery <sup>R</sup> , vector for construction of <i>L. plantarum</i> gene replacement mutants, dual selection suicide vector for <i>L. plantarum</i> ,	Peter Bron, unpublished
pAL0373	Cm <sup>R</sup> Ery <sup>R</sup> , pNZ7101 derivative containing flanking regions of <i>L. plantarum</i> WCFS1 lp_0373	this work
pAL1229	Cm <sup>R</sup> Ery <sup>R</sup> , pNZ7101 derivative containing flanking regions of <i>L. plantarum</i> WCFS1 lp_1229	this work
pCR-Blunt	Kan <sup>R</sup> , cloning vector	Invitrogen
pCR-Blunt 0373	Kan <sup>R</sup> , pCR-Blunt derivative containing <i>L. plantarum</i> WCFS1 lp_0373	this work
pCR-Blunt 1229	Kan <sup>R</sup> , pCR-Blunt derivative containing <i>L. plantarum</i> WCFS1 lp_1229	this work
pJL22	Cm <sup>R</sup> , vector containing constitutive promoter upstream of GUS reporter gene; expression vector for <i>L. plantarum</i> , derived from pNZ123	unpublished
pAW002	Cm <sup>R</sup> , pJL22 derivative containing <i>L. plantarum</i> WCFS1 lp_0373	this work
pAW005	Cm <sup>R</sup> , pJL22 derivative containing <i>L. plantarum</i> WCFS1 lp_1229	this work

Cm<sup>R</sup>, chloramphenicol resistant; Ery<sup>R</sup>, erythromycin resistant; Kan<sup>R</sup>, kanamycin resistant

#### 5 **Example 4 - LP\_1229, protein analysis**

LP\_1229 is a high molecular weight surface protein (1010 amino acids) that has a multi-domain structure, which is typical for cell-surface proteins. Moreover, LP\_1229 contains several repeated domains, present in both the N- and C-terminal parts of the protein. Homology searches reveals the similarity of LP\_1229 to several surface proteins of different lactobacilli and staphylococci, and includes a protein of *Lactobacillus reuteri* that has been shown to be involved in mucus binding properties displayed by this species (Roos and Jonsson 2002). However, the similarities found are limited to specific domains of the LP\_1229 protein and are particularly prominent in the aforementioned repeated domains.

These results show that lp\_1229 is responsible for the mannose-specific adhesion phenotype and therefore elucidates the underlying molecular mechanism of a specific health-benefit mediated by *L. plantarum* and the mutants constructed (both knock-out and overexpression) and provides the tools to modify and exploit this gene, protein and phenotype *in vivo*. The findings also allow for the first time to screen strains and strain collections for the presence and or expression of this functional property and the selection of probiotic strains.

1. An isolated nucleic acid molecule comprising a nucleotide sequence encoding a mannose-specific adhesin, whereby the nucleotide sequence is selected from the group consisting of:
- 5 (a) nucleotide sequences encoding a polypeptide comprising an amino acid sequence that has at least 60 % sequence identity with the amino acid sequence of SEQ ID NO. 1;
- 10 (b) nucleotide sequences comprising a nucleotide sequence that has at least 60% sequence identity with the nucleotide sequence of SEQ ID NO. 2;
- (c) nucleotide sequences the complementary strand of which hybridises to a nucleic acid molecule sequence of (a) or (b);
- (d) nucleotide sequences the sequence of which differs from the sequence of a nucleic acid molecule of (c) due to the degeneracy of the genetic code.
- 15 2. An isolated nucleic acid molecule according to claim 1, wherein the nucleotide sequence encodes a variant or fragment of the mannose-specific adhesin and whereby the variant or fragment is capable of binding mannose.
- 20 3. A vector comprising a nucleic acid molecule according to claim 1 or 2.
4. A vector according to claim 3, wherein the nucleic acid molecule is operatively linked to at least one regulatory DNA element allowing the expression of said nucleic acid in a prokaryotic or a eukaryotic cell.
- 25 5. A host cell comprising a nucleic acid molecule as defined in claim 1 or 2, or a vector as defined in claim 3 or 4.
- 30 6. A host cell according to claim 5, whereby the host cell is a Gram-positive bacterium.

7. A host cell according to claim 6, whereby the host cell belongs to a genus selected from the group consisting of *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Carnobacterium*, *Bifidobacterium*, *Pediococcus*, *Bacillus*, *Streptococcus*.
- 5 8. A host cell according to any one of claims 5 – 7, whereby the nucleic acid molecule confers to the host cell the ability to adhere to human cells.
9. A composition comprising a host cell as defined in claims 5 or 6 and a pharmaceutically or physiologically acceptable carrier.
- 10 10. An isolated polypeptide having an amino acid sequence that has at least 60 % sequence identity with the amino acid sequence of SEQ ID NO. 1.
11. A polypeptide which is a fragment or a variant of the polypeptide of claim 10, 15 whereby the fragment or variant is capable of binding mannose.
12. A composition comprising a polypeptide as defined in claims 10 or 11 and a pharmaceutically or physiologically acceptable carrier.
- 20 13. A method for producing a polypeptide as defined in claims 10 or 11, the method comprising the steps of:
- a) culturing a host cell as defined in any one of claims 5 – 8 under condition conducive to the expression of the polypeptide; and,
- b) recovery of the polypeptide.
- 25 14. A method for producing the composition of claim 12, the method comprising the steps of claim 13 and further comprising the step of mixing the polypeptide with a pharmaceutically or physiologically acceptable carrier.
- 30 15. A method for producing the composition of claim 9, the method comprising the steps of
- a) culturing a host cell as defined in any one of claims 5 – 8; and,
- b) mixing the host cell with a pharmaceutically or physiologically acceptable carrier.

16. A method for treating or preventing a bacterial infection of the gastrointestinal tract, the urinary tract, or the vagina comprising administering to a patient in need of such treatment an effective amount of a composition as defined in claims 9 or 12.
5. 17. A method according to claim 16 whereby the bacterial infection is caused by a bacterium which expresses type 1 fimbriae.
18. Use of a host cell as defined in any one of claims 5 – 8, or a polypeptide as defined in claims 10 or 11 for the manufacture of a medicament for the treatment or prevention of a bacterial infection of the gastrointestinal tract, the urinary tract, or the vagina.
- 10 19. A use according to claim 18, by the bacterial infection is caused by a bacterium which expresses type 1 fimbriae.
- 15 20. A method for determining whether a bacterium has the ability to bind mannose, whereby the method comprises determining the presence or absence of a nucleotide sequence as defined in claim 1 and whereby the presence of the nucleotide sequence is indicative of the ability to bind mannose.
- 20

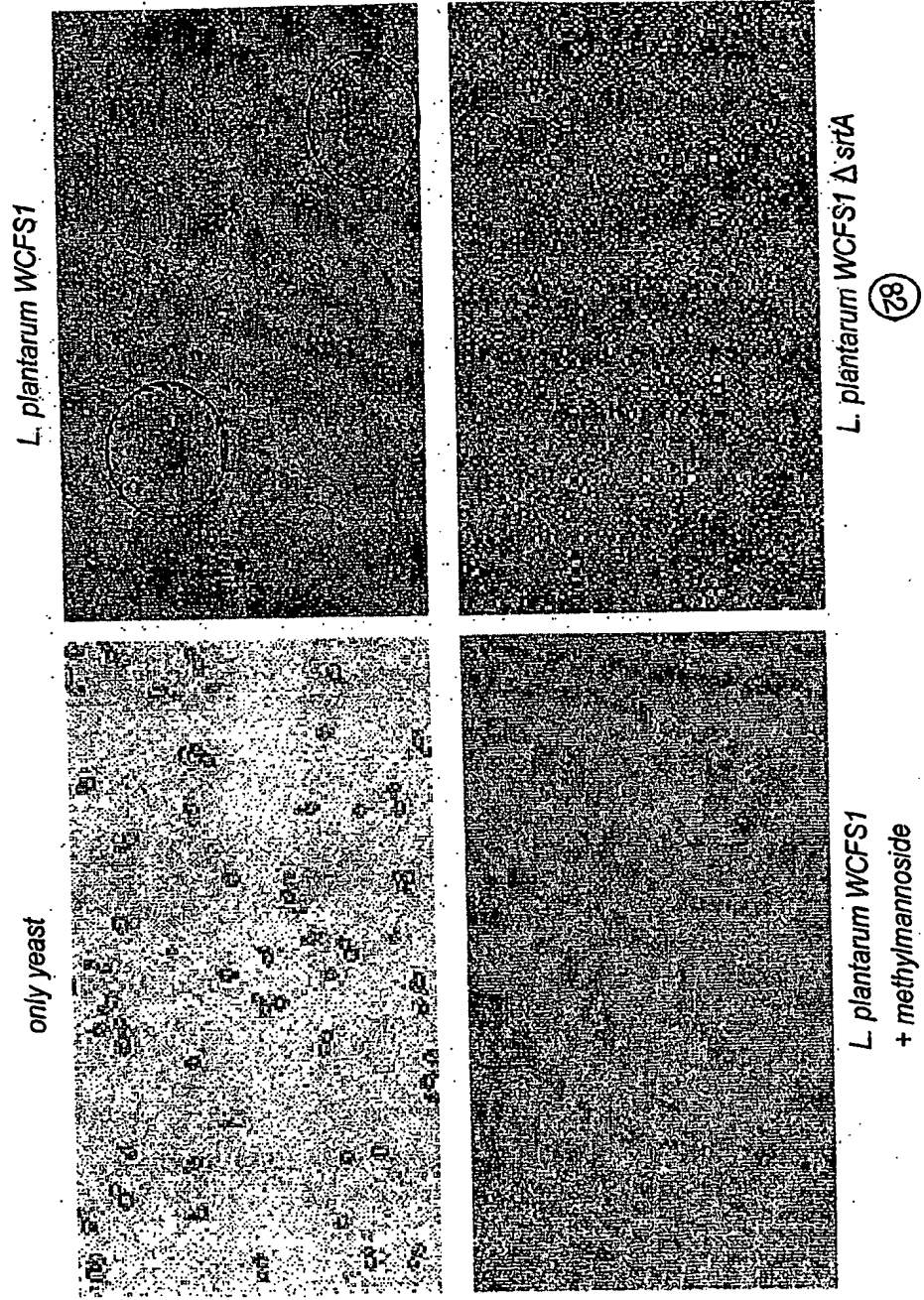
Abstract

The present invention relates to novel mannose-specific adhesins, and variants thereof, as well as nucleic acid sequences encoding these. The invention also relates to host cells expressing nucleic acids encoding the mannose-specific adhesins or variants thereof, as well as to pharmaceutical or nutraceutical compositions comprising the  
5 mannose-specific adhesins and/or host cells expressing the adhesins, and their use in treating or preventing bacterial infection. Further, screening methods suitable for identifying bacteria strains with probiotic properties are provided.

EPO-DG 1  
23.03.2004  
(82)

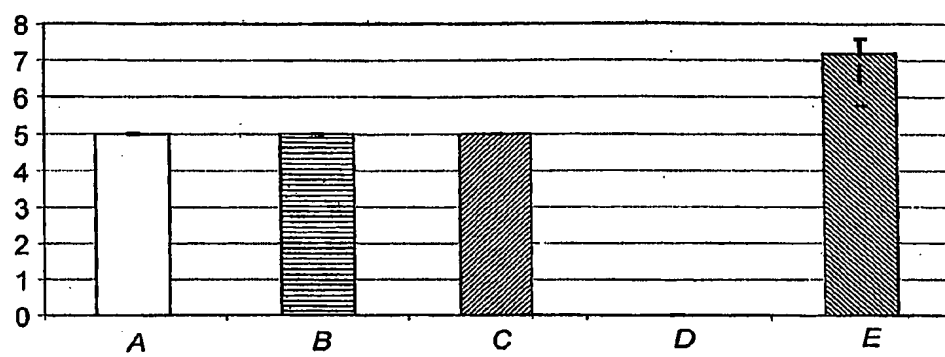


Fig 1



EPO-DG1  
23. 03. 2004

(82)

*Fig 2*

## SEQUENCE LISTING

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10 <120> Novel mannose -specific adhesins and their use

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